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Regulation of *hrp* Genes and Type III Protein Secretion in *Erwinia amylovora* by HrpX/HrpY, a Novel Two-Component System, and HrpS

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Two novel regulatory components, *hrpX* and *hrpY*, of the *hrp* system of *Erwinia amylovora* were identified. The *hrpXY* operon is expressed in rich media, but its transcription is increased threefold by low pH, nutrient, and temperature levels—conditions that mimic the plant apoplast. *hrpXY* is autoregulated and directs the expression of *hrpL*; *hrpL*, in turn, activates transcription of other loci in the *hrp* gene cluster (Z.-M. Wei and S. V. Beer, J. Bacteriol. 177:6201-6210, 1995). The deduced amino -acid sequences of *hrpX* and *hrpY* are similar to bacterial two-component regulators including VsrA/VsrD of *Pseudomonas (Ralstonia) solanacearum*, DegS/DegU of *Bacillus subtilis*, and UhpB/UhpA and NarX/NarP, NarL of *Escherichia coli*. The N-terminal signal-input domain of HrpX contains PAS domain repeats. *hrpS*, located downstream of *hrpXY*, encodes a protein with homology to WtsA (HrpS) of *Erwinia (Pantoea) stewartii*, HrpR and HrpS of *Pseudomonas syringae*, and other σ^{54}-dependent, enhancer-binding proteins. Transcription of *hrpS* also is induced under conditions that mimic the plant apoplast. However, *hrpS* is not autoregulated, and its expression is not affected by *hrpXY*. When *hrpS* or *hrpL* were provided on multicopy plasmids, both *hrpX* and *hrpY* mutants recovered the ability to elicit the hypersensitive reaction in tobacco. This confirms that *hrpS* and *hrpL* are not epistatic to *hrpXY*. A model of the regulatory cascades leading to the induction f the *E. amylovora* type III system is proposed.

Additional keywords: fire blight, pathogenicity, virulence.

Erwinia amylovora is the causal agent of the fire blight disease of many rosaceous plants including pear and apple (van der Zwet and Beer 1999). The bacterium infects blossoms, leaves, succulent shoots, and immature fruits. Symptoms of the infected plants include water soaking and discoloration, followed by necrosis. Sometimes the disease kills whole trees or substantial portions, resulting in devastating economic loss. In nonhost plants such as tobacco and Arabidopsis, the bacterium elicits the defensive hypersensitive reaction (HR), which is characterized by rapid, localized, cell death (Goodman and Novacky 1994). For infection and HR induction, genes generally called *hrp* (hypersensitive response and pathogenicity; see Alfano and Collmer 1996 for a review) are essential.

The *hrp* gene cluster of *E. amylovora* Ea321 has been cloned in several cosmids and enables nonpathogenic bacteria such as *Escherichia coli* to elicit the HR in plants (Beer et al. 1991). According to phenotypic analyses of mutants, *hrp* genes of *E. amylovora* are localized within a 25-kb region of DNA, consisting of at least eight transcriptional units (Wei and Beer 1993). Sequence analysis (Bogdanove et al. 1996; Kim et al. 1997) indicated that the majority of *hrp* genes encode proteins that are thought to be components of a specialized protein secretion apparatus called the type III pathway (Hrp pathway for plant pathogens) (Galán and Bliska 1996). Several proteins including harpins (HrpN and HrpW) and a pathogenicity/avirulence protein (DspE) have been shown to be secreted via the pathway (Bogdanove et al. 1998a; Kim and Beer 1998; Wei and Beer 1993).

Transcriptional expression of *hrp* genes is induced under conditions similar to the environment of the plant apoplast: low carbon and nitrogen, low pH (5.5), and low temperature (18°C) (Wei et al. 1992). Two independent loci, complementation groups IV and V, in the *hrp* cluster were found to have regulatory function (Sneath et al. 1990; Wei and Beer 1993, 1995). Mutations in these loci abolish harpin production and the HR-eliciting and disease-causing abilities of *E. amylovora* (Wei and Beer 1993). Preliminary sequence analysis indicated that one of them (group IV) contains a gene called *hrpS* (Sneath et al. 1990) that encodes a protein similar to σ^{54}-dependent transcriptional activators (Morett and Segovia 1993). Complementation group V encodes *hrpL* (Wei and Beer 1995), which is homologous to genes encoding members of the ECF subfamily of eubacterial sigma factors (Lonetto et al. 1994). HrpL recognizes conserved promoter sequences called "*hrp* boxes" (Xiao and Hutcheson 1994), and directs the transcription of other pathogenicity genes including *hrp* secretion operons (*hrpA*, *hrpC*, and *hrpJ*) (Wei and Beer 1995), harpin genes (*hrpN* and *hrpW*) (Kim and Beer 1998; Wei and Beer 1995), and a disease-specific locus (*dspEF* [Bogdanove et al. 1998b]; *dspAB* [Gaudriault et al. 1997]).

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Here we report the characterization of two new regulatory genes, designated *hrpX* and *hrpY*, and the further analysis of *hrpS*. *hrpX* and *hrpY* are present in an operon situated between *hrpS* and *hrpL*. Analysis f deduced protein sequences suggested that they constitute a two-component regulatory complex; HrpX functioning as a sensor and HrpY as the response-regulator partner of HrpX. *hrpX*, *hrpY*, and *hrpS* are components of a complex regulatory network that leads to activation of *hrpL* and eventually other genes in the *hrp* cluster of *E. amylovora*.

RESULTS

Identification and sequence analysis of the *hrpXY* locus.

Previous studies have identified several loci, including *hrpC*, *hrpA*, *hrpS*, *hrpL*, and *hrpJ*, that are essential for the Hrp phenotype (Bogdanove et al. 1996; Kim et al. 1997; Wei and Beer 1993, 1995) (Fig. 1A). Preliminary genetic analysis of pCPP430 in *Escherichia coli* suggested the presence of a new locus, between *hrpS* and *hrpL*, that also is required for the Hrp phenotype and contains novel regulatory components. We have designated this locus *hrpXY*.

A 3.4-kb *Bgl*II- and *Cla*I-digested fragment of pCPP430 was cloned into pBluescript KS+, resulting in pCPP1178. The sequence of the insert of pCPP1178 revealed two tightly linked open reading frames (ORFs) between *hrpL* and *hrpS* that are capable of encoding proteins of 495 and 213 amino acid residues, respectively (Fig. 1B). These ORFs were named *hrpX* and *hrpY*, respectively. Potential ribosome-binding sites, AGGAG and TGGAA, were found 5 and 7 bp upstream of the *hrpX* and *hrpY* start codons, respectively. Although the ribosome-binding site ahead of *hrpY* weakly matches the consensus sequence, we assume it is sufficient for translation of *hrpY*; only a 4-bp space exists between the *hrpX* stop codon and *hrpY* start codon and translational coupling is plausible. To confirm that the *hrpX* and *hrpY* ORFs produce proteins, pCPP1178 was placed in a gene expression system mediated by the T7 RNA polymerase. Two distinct protein bands were visible following sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). The apparent molecular masses of HrpX and HrpY were about 50 and 25 kDa, respectively (data not shown), close to the sizes expected from the deduced amino acid sequences.

The start codon of *hrpX* is located 146 bp downstream of the *hrpL* stop codon, and a promoter prediction program (see Materials and Methods) identified two putative σ^{70} promoter sequences, TAGACG-N_{17}-TAAAGT (score from promoter prediction by neural network = 0.97) and TTGCAA-N_{16}-CCTAAT (score = 0.95), 111 and 33 bp upstream of the *hrpX* start codon, respectively. There is a 361-bp noncoding region between *hrpY* and *hrpS*. Palindromic sequences that may serve either as targets of regulatory components or as transcription terminators, GTAAACANTGTTTAC and GGATAAAATGG-TTGTGG-N_7-CCGCTTCCATTTTATCC, were identified in the *hrpL-hrpX* and *hrpY-hrpS* intergenic regions, respectively. The tight linkage of *hrpX* and *hrpY*, and the existence of long noncoding areas and inverted repeats upstream of *hrpX* and downstream of *hrpY*, suggest that the two genes form an operon.

HrpX and HrpY constitute a two-component regulatory system.

Comparison of the predicted amino acid sequences of *hrpX* and *hrpY* with sequences in the data bases revealed significant similarities with many two-component regulatory proteins. The homologs include VsrA/VsrD of *Pseudomonas* (now *Ralstonia*) *solanacearum*, which regulate virulence gene expression (Huang et al. 1995b); UhpB/UhpA of *Escherichia coli*, which participate in the regulation of sugar transport (Friedrich and Kadner 1987); NarX/NarP,NarL of *Escherichia*

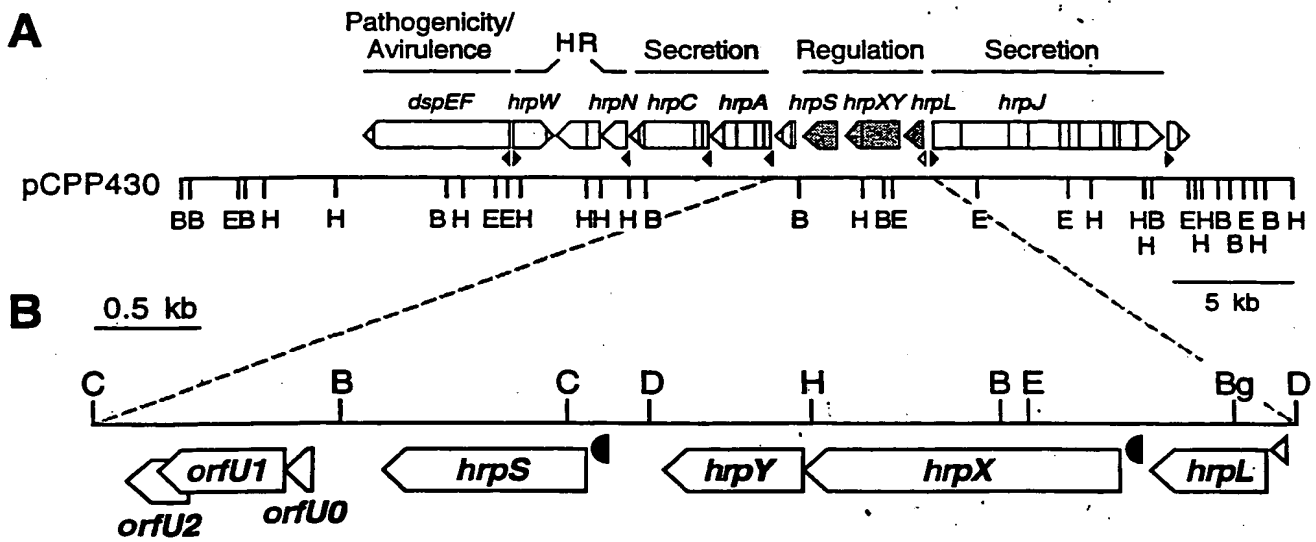

Fig. 1. A, Operon organization of the *hrp/dsp* gene cluster of *Erwinia amylovora* cloned in pCPP430. B, Central region covering regulatory genes *hrpL*, *hrpX*, *hrpY*, and *hrpS*. Boxes and arrow boxes: transcriptional units or open reading frames; names of the characterized operons or genes are given above, inside, or below. Filled triangles: putative HrpL-dependent promoters. Open triangles: putative σ^{54} promoters. Closed half circles: putative σ^{70} promoters. Restriction enzymes: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; Bg, *Bgl*II; C, *Cla*I; and D, *Dra*I.

coli, which are involved in the regulation of anaerobic respiratory gene expression (Rabin and Stewart 1993); and DegS/DegU of *Bacillus subtilis*, which are involved in extracellular enzyme production (Kunst et al. 1988) (Fig. 2; Table 1). In addition, HrpY showed high sequence similarity with many ther transcriptional activators including ExpA of *E. carotovora* (33% identity), which is involved in global control of virulence (Eriksson et al. 1998); UvrY f *Escherichia coli* (33% identity) (Sharma et al. 1986); SirA of *Salmonella typhimurium* (32% identity) (Johnston et al. 1996); and GacA of several animal- and plant-associated *Pseudomonas* spp. (29 to 30% identities) (Laville et al. 1992).

The high sequence similarity of HrpX with histidine kinases suggests that HrpX is a sensor. HrpX has the conserved His

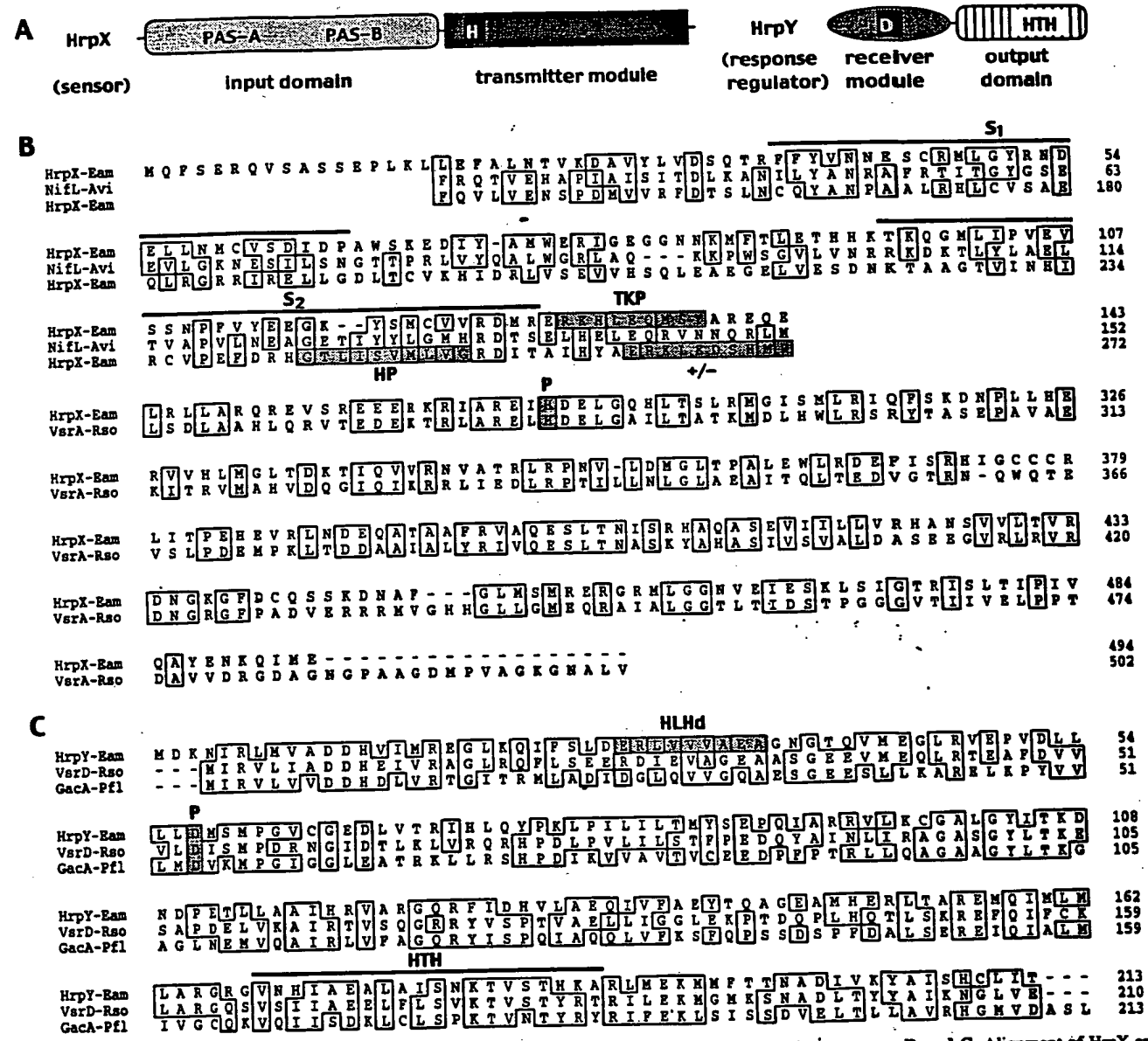

Fig. 2. A, Diagramatic illustrations of HrpX and HrpY of *Erwinia amylovora* showing predicted domain structures. B and C, Alignment of HrpX and HrpY with similar proteins. Designations of diagrams are after Parkinson and Kofoid (1992). PAS-A and PAS-B denote two repeats of the PAS domain, H and D phosphorylated histidine and aspartate residues, respectively, and HTH the helix-turn-helix DNA-binding motif. Overlines represent the S-boxes in the PAS domain (S_1 and S_2) and the HTH motif. A putative tyrosine kinase phosphorylation site (TKP), a hydrophobic region (HP), a putative charge-rich linker (+/–), and a helix-loop-helix dimerization domain signature (HLHd), and phosphorylation sites (P) are shown by shading. PILEUP program (GCG software package, vers. 7.3; Genetics Computer Group, Madison, WI, U.S.A.) with default parameters was used to align the sequences. Accession numbers of the compared proteins: NifL of *Azotobacter vinelandii*, SWISS-PROT:P30663; VsrA and VsrD of *Ralstonia solanacearum*, PIR:S41544 and PIR:I40540; and GacA of *Pseudomonas fluorescens*, SWISS-PROT:P32967. In the HrpX alignment, only 27 to 152 residues of NifL and C-terminal 243 residues of VsrA are shown. Also, the HrpX sequence is shown on two lines for the first three rows.

residue for autophosphorylation and a hydrophobic domain that may enable the protein to be transiently associated with the cytoplasmic membrane (Fig. 2B). The C-terminal putative transmitter domain (residues 273 to 494) of HrpX shows most similarity to the kinase domains of the sensor proteins listed in Table 1; the N-terminal putative input domain of HrpX shows similarity to PAS domains (Zhulin et al. 1997) of *Methanobacterium thermoautotrophicum*, *Azotobacter vinelandii*, and other organisms. Several PAS-containing proteins are sensors of bacterial two-component systems. The PAS domain typically consists of two direct sequence repeats (PAS-A and PAS-B), and each repeat contains two highly conserved regions called S_1 and S_2 boxes (Zhulin et al. 1997). In the case of HrpX, the second repeat (PAS-B) seems imperfect (Fig. 2B). Based on ScanProsite analysis (Appel et al. 1994), another feature of HrpX with unknown functional relevance is a putative tyrosine kinase phosphorylation site (PROSITE:PS00007).

HrpY appears to be a response regulator with a putative receiver domain at the N terminus (up to 102 amino acid residues) and a DNA-binding domain at the C terminus (Fig. 2A). As shown in Figure 2C, HrpY contains the conserved Asp residue, which may be phosphorylated by the sensor, and the helix-turn-helix DNA binding motif. HrpY also has a sequence that matches the Myc-type helix-loop-helix dimerization domain signature (PROSITE:PS00038), the functional significance of which remains to be tested.

Table 1. HrpX and HrpY of *Erwinia amylovora* compared with two-component regulatory proteins (sensors/response regulators) of other bacteria

Bacterium	Protein	Amino acids	% Identity[a]
Erwinia amylovora	HrpX/HrpY	494/213	–
Ralstonia solanacearum	VsrA/VsrD	502/210	34/41
Escherichia coli	UhpB/UhpA	500/196	32/32
Bacillus subtilis	DegS/DegU	385/229	32/28
Escherichia coli	NarX/NarP, NarL	598/215, 216	31/33, 32

[a] % Identities from a BLASTP search of HrpX and HrpY with default parameters, except for no filtering for low complexicity regions. Only the transmitter domain of HrpX (residues 273 to 494) was used for comparisons with other sensor proteins.

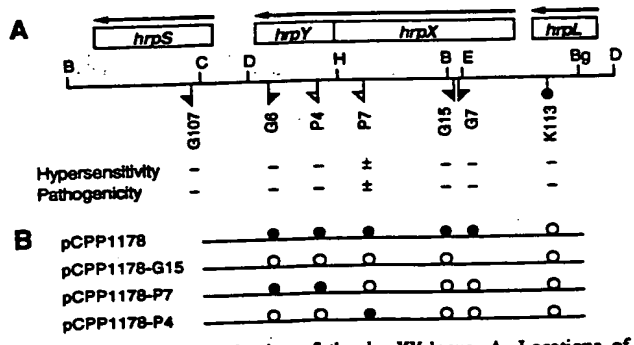

Fig. 3. Genetic characterization of the *hrpXY* locus. A, Locations of transposon insertions and phenotypes of *hrpX* and *hrpY* mutants of *Erwinia amylovora* Ea321. Rectangles above map of restriction enzymes and transposons represent transcriptional units. Arrows: directions of transcription. Closed flags: insertions by Tn*5-gusA1*. Open flags: insertions by Tn*phoA*. Llollipop: a Tn*10*-miniKm insertion. Mutants shown by minus signs below insertion points did not elicit the hypersensitve reaction (HR) or cause disease (Hrp⁻); a mutant shown by ± infrequently elicited spotty HR and showed low virulence. B, Complementation assay of *hrpX* and *hrpY* mutants of *E. amylovora* Ea321 with various plasmids. Closed circle: plasmid complemented Hrp phenotype of the mutant containing the transposon insertion in the same column. Open circle: plasmid did not change the phenotype of corresponding mutant.

Genetic characterizati n f *hrpX* and *hrpY*.

The *hrpXY* locus in pCPP430 was mutagenized with transposons Tn*5-gusA1* and Tn*phoA*. Derivatives of pCPP430 containing the transposon insertions were marker-exchanged into the genome of *E. amylovora* Ea321. All *hrpY* mutants of Ea321 failed to elicit the HR in tobacco and to infect immature pear fruits (Fig. 3A). Two classes of *hrpX* insertion mutants were obtained. Ea321-G15 and Ea321-G7, which were made with Tn*5-gusA1*, were similar to *hrpY* mutants in phenotypes. Ea321-P7, an *hrpX*::Tn*phoA* mutant, caused slight tissue collapse in tobacco at higher inoculum dose and had low virulence in immature pears, rather than the strict Hrp⁻ phenotype (Fig. 3A). Specifically, tobacco leaves infiltrated with Ea321-P7 at $\geq 5 \times 10^8$ CFU per ml developed a spotty HR 36 h after infiltration. Also, in immature pears inoculated with the mutant, bacterial ooze appeared 3 days later than in those inoculated with the wild type, and the population of the mutant recovered was only one-tenth of that of the wild type (data not shown).

Virulence of the mutants was restored to near wild-type levels by providing the mutants with pCPP1178 in *trans* (Fig. 3B). The *hrpX*::Tn*5-gusA1* mutants of Ea321 were not complemented by pCPP1178-P4 that contains a transposon insertion in *hrpY* (Fig. 3B). This suggests that *hrpX* and *hrpY* are in the same transcriptional unit and the Tn*5-gusA1* mutations in *hrpX* are polar. We found, however, that the *hrpX*::Tn*phoA* mutant Ea321-P7 can be complemented by pCPP1178-P4, indicating that the Tn*phoA* insertion of *hrpX* did not affect the function of *hrpY* (Fig. 3B). Tn*phoA*-induced mutations that permit the expression of downstream genes have been observed frequently in *E. amylovora* (Z. Wei and S. V. Beer, *unpublished data*) and *Pseudomonas syringae* (Huang et al. 1995a). Thus, we believe that the P7 insertion is nonpolar and that the peculiar phenotype of the Ea321-P7 may reflect the function of *hrpX*.

All the transposon mutations in the *hrpXY* locus were complemented by derivatives of pCPP430 with transposon insertions in *hrpS* or *hrpL* (data not shown), confirming the suggestion from sequence analysis that *hrpX* and *hrpY* constitute an independent complementation group. Based on results of sequence analysis and genetic characterization, we conclude (i) *hrpXY* is required for the Hrp phenotype, and (ii) *hrpX* and *hrpY* constitute a two-gene operon, *hrpXY*.

Expression of *hrpXY* is environmentally regulated.

A new construct, pCPP1203, was used to monitor expression of the *hrpXY* promoter in a nutrient-rich medium and a minimal medium that induces the expression of *hrp* genes (Wei et al. 1992). pCPP1203 was derived from pCPP1178-G15 (*hrpX*::Tn*5-gusA1*) in which the directions of *hrpX* and *gusA* are the same. pCPP1178-G15 was digested with *Bam*HI and *Sac*I (an *Sac*I site is present in the vector), which cuts out the *hrpXY* promoter region, a 5′ portion of the *hrpX* coding region fused to Tn*5-gusA1*, and the whole transposon. The resulting fragment was then ligated to pCPP43, which had been digested with the same enzymes. pCPP43 (gift of David

W. Bauer) is a derivative of pOU61, which is a low-copy-number plasmid (approximately one copy per bacterium at 30°C) (Larsen et al. 1984).

In *E. amylovora* and *Escherichia coli*, the *hrpXY* promoter directed high levels of basal expression in Luria broth (LB), but expression of *hrpX*::Tn*5-gusA1* was enhanced threefold in the *hrp*-inducing minimal medium (IM) (Table 2). Enhanced levels of *hrpX*::Tn*5-gusA1* expression were also observed from assays of the strains in tobacco leaves and immature pears (data not shown). No β-glucuronidase (GUS) activity was detected for *Escherichia coli* SØ200Δ*uidA*(pCPP1203) unless functional *hrpXY* was provided (Table 2). Similarly, high basal-level expression of *hrpX*::Tn*5-gusA1* of Ea321(pCPP1203) in Table 2 is probably due to functional *hrpXY* present in the chromosome. The latter two observations indicate that *hrpXY* is also autoregulated.

hrpX and *hrpY* control the expression of *hrpL*.

To study the effect of *hrpX* and *hrpY* on the control of *hrpL* expression, a *hrpL*::Tn*5-gusA1* fusion (pCPP139-G44) (Wei and Beer 1995) was marker exchanged into an *hrpX* mutant (Ea321-P7) and an *hrpY* mutant (Ea321-P4), to generate *hrpX-hrpL* and *hrpY-hrpL* double mutants Ea321-P7G44 and Ea321-P4G44, respectively. Mutation in *hrpY* completely abolished *hrpL* expression (Fig. 4). However, the *hrpX* mutant reduced *hrpL* expression only to about 20% of its wild-type level, opening the possibility that the mutated HrpX may be still partially functional or another sensor protein may cross talk with HrpY.

Analysis of the *hrpS* locus and the ORFs between *hrpS* and *hrpA*.

hrpS also partially controls *hrpL* expression in *E. amylovora* and is located downstream of *hrpXY* (Wei and Beer 1995). We report here the entire nucleotide sequence of the region between *hrpY* and *hrpA*, which includes *hrpS*, to complete the preliminary results on *hrpS* presented previously (Sneath et al. 1990).

The *hrpS* locus of *E. amylovora* Ea321 contains a single-gene operon, based on the large intergenic regions beyond the coding region of *hrpS*, and a potential terminator, CGGCGACAGC-N_5-GCTGTCGCCG, that lies 49 bp downstream of the *hrpS* stop codon. The *hrpS* ORF is preceded by a potential σ^{70} promoter, GTGGCA-N_{18}-TATTAC (score from promoter prediction by neural network = 0.96), and it encodes a 324 amino acid protein. HrpS has homology to members of the σ^{54}-dependent, enhancer-binding protein family (Morett and Segovia 1993). HrpS shows highest sequence similarity with WtsA (HrpS) of *Erwinia* (*Pantoea*) *stewartii* (Frederick et al. 1993) (79% identity over 322 amino acid residues without gaps from BLASTP), HrpR and HrpS of *P. syringae* pathovars (51 to 55% identities) (Grimm et al. 1995; Xiao et al. 1994), and DctD of *Rhizobium* spp. (39% identities) (Jiang et al. 1989; Ronson et al. 1987). HrpS of *E. amylovora* has two putative ATP-binding sites at the N terminus and a helix-turn-helix DNA-binding motif at the C terminus (Fig. 5A). HrpS shows high sequence similarity to other regulators in the NtrC family throughout the entire σ^{54} interaction domain. However, similar to other HrpR/HrpS proteins, HrpS of *E. amylovora* contains a very short N-terminal A domain (Shingler 1996), and seems to lack the phosphorylation receiver domain (Fig. 5A).

In the region between *hrpS* and *hrpA*, three potential genes, designated *orfU0*, *orfU1*, and *orfU2* (Fig. 1B), were identified by application of the GeneMark.hmm algorithm (Lukashin and Borodovsky 1998). *orfU0* is a small ORF encoding a 46 amino acid basic protein, without significant similarity to any protein in the data base. Preceded by GGAGT 8 bp upstream, *orfU1* encodes a 203 amino acid basic protein that is similar to a conserved hypothetical protein HP1401 of *Helicobacter pylori* (32% identity over 164 amino acid residues with 12 gaps) (Fig. 5B). Interestingly, protein sequence of the next ORF, *orfU2*, shows even higher similarity to HP1401 (residues 189 to 229; 41% identity without gaps). This suggests the possibility that a frame shift in *orfU1-orfU2* resulted in the two current ORFs, and that both may be defective. The lack of an obvious promoter in front of *orfU0*, the lack of good ribosome-binding sites in front of *orfU0* and *orfU2*, the potential frame-shift mutation at the 3′ region of *orfU1*, and the lack of a phenotype of Tn*phoA*-induced *orfU1* mutants (data not shown) indicate that the region comprising *orfU0-orfU2* is unlikely to be functional in Ea321.

Expression of *hrpS* is not autoregulated, and induction of *hrpS* is independent of *hrpX* or *hrpY*.

An *hrpS*::*gusA1* fusion designated G107 (Wei et al. 1992) was used to assay the expression of *hrpS*. A fragment of

Table 2. Expression of the *hrpXY* promoter in Luria broth (LB) and in a *hrp*-inducing minimal medium (IM)

Bacterial strain[a]	GUS activity[b]	
	LB	IM
Erwinia amylovora Ea321(pCPP1203)	242 ± 12	788 ± 32
E. coli SØ200Δ*uidA*(pCPP1203)	2 ± 3	3 ± 3
E. coli SØ200Δ*uidA*(pCPP1203, pCPP1178)	145 ± 19	878 ± 33

[a] *E. coli* SØ200Δ*uidA* is an *Escherichia coli* strain with no β-glucuronidase (GUS) activity due to deletion of *gusA*. pCPP1203 is a low-copy-number plasmid containing *hrpX*::Tn*5-gusA1*; pCPP1178 is a high-copy-number plasmid containing functional *hrpX* and *hrpY* genes.
[b] Picounits per CFU; mean of three replicates ± standard deviation.

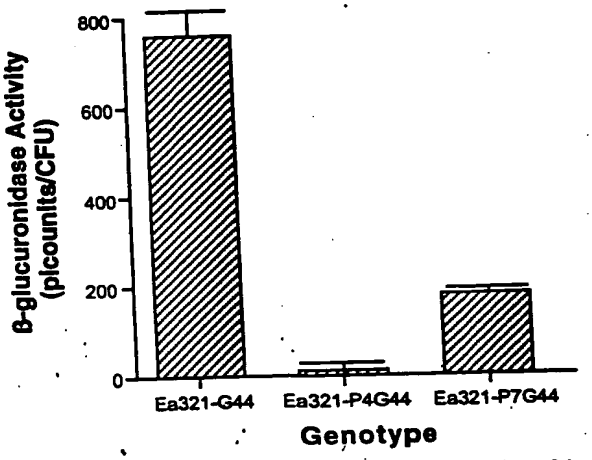

Fig. 4. Effect of mutations in *hrpX* and *hrpY* on expression of *hrpL*. Genotypes of the strains are Ea321-G44, *hrpL*::Tn*5-gusA1* (Wei and Beer 1995); Ea321-P4G44, *hrpY*::Tn*phoA* and *hrpL*::Tn*5-gusA1*; and Ea321-P7G44, *hrpX*::Tn*phoA* and *hrpL*::Tn*5-gusA1*. Error bars: standard deviation from three replicates. Cells grown in inducing medium (IM) were assayed (see Materials and Methods for details).

pCPP430-G107 digested with *Bam*HI contains the whole transposon, the *hrpS* gene fused to Tn*5-gusA1*, and the *hrpS* promoter region. This *Bam*HI fragment was ligated with a low-copy-number plasmid, pCPP8 (Bauer 1990), that was cut with the same enzyme. The resulting plasmid was designated pCPP1058. As with *hrpXY*, expression of *hrpS* in *Escherichia coli* or in *E. amylovora* was induced under *hrp*-inducing conditions (Table 3). However, autoregulation was not required for *hrpS* expression; the presence of functional *hrpS* did not affect the expression of a *hrpS*::*gusA1* fusion in pCPP1058 (Table 3).

To determine whether the newly discovered two-component system has any effect on the expression of *hrpS*, an *hrpS*::Tn*5-gusA1* fusion (pCPP430-G107) was marker-exchanged into *hrpX* and *hrpY* mutants. Neither *hrpX* nor *hrpY* affected *hrpS* expression significantly (Fig. 6).

hrpS and *hrpL*, provided by multicopy plasmids, suppress defects in *hrpX* or *hrpY*.

To further characterize the regulatory relationships between *hrpXY*, *hrpS*, and *hrpL*, the HR-impaired strains Ea321-P7, Ea321-P4, and Ea321-G107 were transformed with pCPP1178

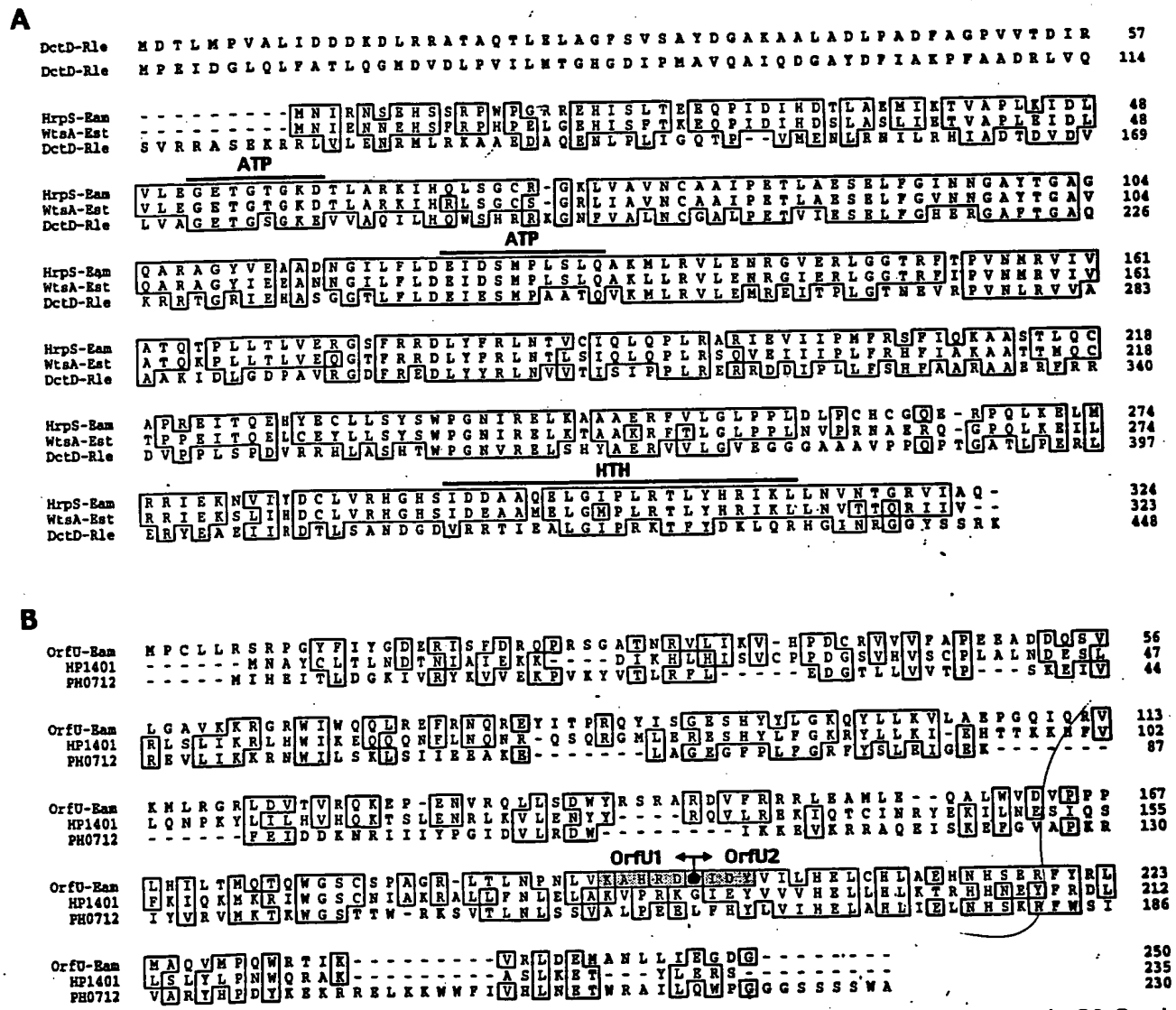

Fig. 5. Alignments of A, HrpS, and B, OrfU of *Erwinia amylovora* with similar proteins. PILEUP program (GCG software package, version 7.3; Genetics Computer Group, Madison, WI, U.S.A.) with default parameters was used to align the sequences. Overlines represent ATP-binding sites (ATP) and the helix-turn-helix DNA-binding motif (HTH). Sequence of OrfU is a composite of sequences of *orfU1* and *orfU2* products. A putative tyrosine kinase phosphorylation site (PROSITE: PS00007) is indicated by shading. Black circle in the OrfU sequence denotes location of a probable reading-frame shift. Accession numbers: WtsA of *E. stewartii*, SWISS-PROT:P36219; DctD of *Rhizobium leguminosarium*, SWISS-PROT:P10046; HP1401 of *Helicobacter pylori*, GENBANK:AE000640; and PH0712 of *Pyrococcus horikoshii*, DDBJ:AP000003.

(contains *hrpXY*), pCPP1001 (contains *hrpS*) (Wei and Beer 1995), or pCPP1078 (contains *hrpL*) (Wei and Beer 1995). The resulting transformants were infiltrated into panels f tobacco leaves to determine which, if any, of the regulatory genes, when present in multiple copies, are sufficient to restore the HR-eliciting ability to the mutants. Panels infiltrated with *hrpX* and *hrpY* mutants containing *hrpL* developed the HR (Table 4), often faster than panels infiltrated with the wild-type strain. The panels began to show collapse 8 to 12 h after infiltration; by 24 h, the whole infiltrated area had collapsed in a typical HR. This result is consistent with dependence of *hrpL* expression on *hrpX* and *hrpY*. Interestingly, similar suppression was observed from *hrpX* and *hrpY* mutants containing *hrpS*, whereas *hrpX* and *hrpY* did not restore the HR phen type of the *hrpS* mutant (Table 4).

DISCUSSION

The HrpX/HrpY two-component protein system.

Our results demonstrate that *E. amylovora* employs the HrpX/HrpY two-component regulatory proteins to direct expression of an alternate sigma factor gene, *hrpL*, that in turn activates a type III protein secretion system. This provides for a quick change in the pattern of gene expression needed to initiate infection. HrpX is a putative I_CT-type sensor (Parkinson and Kofoid 1992) composed of the N-terminal PAS domain and the C-terminal histidine kinase domain (Fig. 2A). HrpX appears to be cytoplasmic, and may be anchored to the inner membrane by its internal hydrophobic region. Other members f the PAS-containing I_CT-type sensor kinases include NifL, NtrB, and KinA (Zhulin et al. 1997). HrpY appears to be a RO_M subfamily response regulator (Parkinson and Kofoid 1992). Consistent with the HrpX transmitter domain, HrpY shows significant sequence similarity to VsrD, DegU, UhpA, and NarL.

Two-component systems with PAS domains in the sensor component include NifL/NifA, DctS/DctR, and BvgS/BvgA (Zhulin et al. 1997). Among these only NifL does not contain the periplasmic domain, and HrpX is more similar to NifL than the other two. NifL and most other PAS-containing proteins are sensors (Zhulin et al. 1997), and their signal input domains are located at the N terminus (Parkinson and Kofoid 1992). Thus, HrpX may directly perceive environmental signals with its N-terminal PAS domain. One function of the PAS domain is to act as a protein dimerization motif (Kay 1997). This raises the possibility of HrpX dimerization, which is required for the functional state of two-component sensors (Parkinson and Kofoid 1992).

Two-component regulatory system and type III protein secretion.

Although the two-component system is widely used to control bacterial gene expression (Hoch and Silhavy 1995), reports of its function in regulation of the type III system are just emerging. In *S. typhimurium*, SirA is a response regulator essential for induction of *hilA*, *prgHIJK*, and *sigDE* (Hong and Miller 1998; Johnston et al. 1996), and the PhoQ/PhoP two-component system represses the expression of the *prg* locus (Pegues et al. 1995). The CpxA/CpxR system controls the pH-dependent expression of the *Shigella sonnei virF* gene, which in turn activates *ipaBCD* and *virG* (Nakayama and Watanabe

Table 3. Expression of the *hrpS* promoter in Luria broth (LB) and in *hrp*-inducing minimal medium (IM)

Bacterial strain[a]	GUS activity[b] LB	IM
E. coli SØ200Δ*uidA*(pCPP1058)	94 ± 12	367 ± 9
E. coli SØ200Δ*uidA*(pCPP1058, pCPP1001)	105 ± 17	378 ± 23
Erwinia amylovora Ea321-G107	36 ± 11	188 ± 35
Erwinia amylovora Ea321-G107(pCPP1001)	42 ± 21	229 ± 29

[a] *E. coli* SØ200Δ*uidA* is an *Escherichia coli* strain with no β-glucuronidase (GUS) activity due to deletion of *gusA*. *Erwinia amylovora* Ea321-G107 is a mutant of Ea321 containing a Tn*5-gusA1* insertion in *hrpS* (Wei et al. 1992). pCPP1058 is a low-copy-number plasmid containing *hrpX*::Tn*5-gusA1*; pCPP1001 is a high-copy-number plasmid containing the functional *hrpS* gene and its promoter (Wei and Beer 1995).

[b] Picounits per CFU; meanof three replicates ± standard deviation.

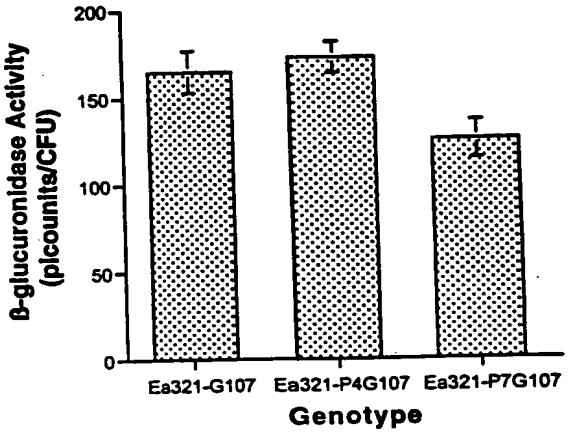

Fig. 6. Effect of mutations in *hrpX* and *hrpY* on expression of *hrpS*. Genotypes of the strains are Ea321-G107, *hrpS*::Tn*5-gusA1* (Wei et al. 1992); Ea321-P4G107, *hrpY*::Tn*phoA* and *hrpS*::Tn*5-gusA1*; and Ea321-P7G107, *hrpX*::Tn*phoA* and *hrpS*::Tn*5-gusA1*. Error bars: standard deviation for three replicates. Cells grown in inducing medium (IM) were assayed (Materials and Methods contains details).

Table 4. Hypersensitive reaction (HR) elicitation by *hrp* regulation mutants

Strain	Genotype	HR phenotype[a]
Ea321	wild type; *hrp*$^+$	+++
Ea321-P7	*hrpX*	±
Ea321-P7(pCPP1178)	*hrpX*(*hrpXY*$^+$)	++[b]
Ea321-P7(pCPP1001)	*hrpX*(*hrpS*$^+$)	+++
Ea321-P7(pCPP1078)	*hrpX*(*hrpL*$^+$)	+++
Ea321-P4	*hrpY*	–
Ea321-P4(pCPP1178)	*hrpY*(*hrpXY*$^+$)	++[b]
Ea321-P4(pCPP1001)	*hrpY*(*hrpS*$^+$)	+++
Ea321-P4(pCPP1078)	*hrpY*(*hrpL*$^+$)	+++
Ea321-G107	*hrpS*	–
Ea321-G107(pCPP1178)	*hrpS*(*hrpXY*$^+$)	–
Ea321-G107(pCPP1001)	*hrpS*(*hrpS*$^+$)	+++

[a] +++, full HR manifested by complete tissue collapse throughout infiltrated area; ++, reduced HR, which is spotty and often coalescing; ±, infrequent collapse and small spotty necreosis for HR-positive leaves; and –, no HR. Inoculum concentration was approximately 2×10^8 CFU per ml. Ratings (consensus of four plants) were made 36 h after inoculation.

[b] Full HR was observed at inoculum levels of $\geq 5 \times 10^8$ CFU per ml.

1995). Also, the BvgS/BvgA system was recently found to be involved in the regulation f the type III secretion in *Bordetella bronchiseptica* (Yuk et al. 1998). Among plant pathogens, HrpG of *Xanthomonas campestris* pv. *vesicatoria*, a hom log of response regulators, has been shown to regulate *hrpXv* and *hrpA* expression (Wengelnik et al. 1996).

The structure of the input domain of *E. amylovora* HrpX appears to be exceptional, compared with sensor proteins involved in other type III systems, which contain two transmembrane regions and a periplasmic domain. The closest homologs of *E. amylovora* HrpY are SirA and BvgA, both of which are RO_{III}-type regulators (Parkinson and Kofoid 1992), whereas *X. campestris* HrpG belongs to the RO_{II} type, which includes *Escherichia coli* CpxR and OmpR, *S. typhimurium* PhoP, and *Agrobacterium tumefaciens* VirG. Thus, at least two types of transmitter-receiver systems appear to have evolved for control of type III systems in response to environmental stimuli in hosts. Also, the two two-component systems identified in the plant pathogens *E. amylovora* and *X. campestris* fall into different communication groups.

HrpS and mechanism of gene regulation.

HrpS is a member of the σ^{54}-dependent, enhancer-binding protein family. Both *hrpS* and *rpoN* are required for transcription f *hrp* genes in *P. syringae* pathovars (Grimm et al. 1995; Xiao et al. 1994). WtsA (HrpS) of *E. stewartii* controls expression of *wtsB*, which also requires the presence of σ^{54} (Frederick et al. 1993). In *E. amylovora*, HrpS partially regulates *hrpL* expression (Wei and Beer 1995), and a sequence, TGGCAC-N_5-TTGC, that perfectly matches the –24/–12 promoter consensus sequence is found at the promoter region of *E. amylovora hrpL*. The *hrpS* gene of *E. amylovora*, but not *hrpS* of *P. syringae* pv. *phaseolicola*, can complement the *hrpS* mutation in *E. stewartii* (Frederick et al. 1993). The HrpS sequences of the two erwinias are highly similar, and even the upstream noncoding regions appear to be conserved, except for the insertion of a 484-bp sequence, reminiscent of an IS (insertion sequence) element, 23-bp upstream of the *E. stewartii hrpS* ORF.

As a member of the NtrC family, HrpS is unusual in that it lacks a long N-terminal receiver domain. Control of protein activation by phosphorylation, by protein-protein interaction, and by signal molecule have been suggested for σ^{54}-dependent proteins (Shingler 1996). In the direct activation model, derepression by effectors seems to be a mechanism of protein activation. For DctD, DmpR, and XylR, deletion of the receiver domain results in constitutive activation of the proteins, suggesting that the receiver domain has a repressor function

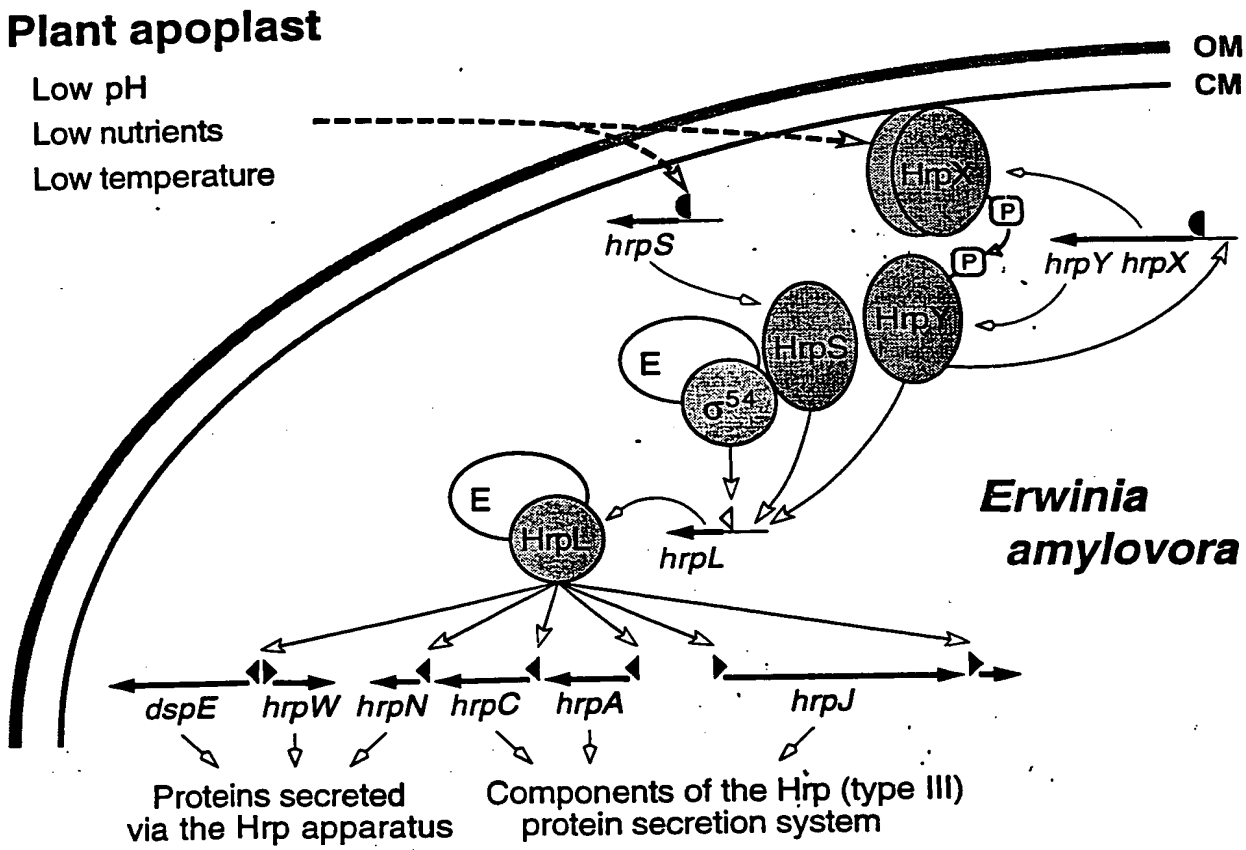

Fig. 7. Model of the *hrp* gene regulatory cascade. Thick arrow lines: genes or operons. Ovals and circles: proteins. Arrowheads in thinner lines: directions of information flow. CM, cytoplasmic membrane; OM, outer membrane; P, phosphate; E, RNA polymerase; closed half circle, σ^{70} promoter; open triangle, σ^{54} promoter; and filled triangle, HrpL promoter.

(Shingler 1996). Therefore, the apparent absence of the receiver domain in HrpS implies that HrpS may not require phosphorylation for activation and is always active once the protein is made.

Induction of *hrpXY* and *hrpS* and the involvement of HrpXY and HrpS in *hrpL* regulation.

Expression of *hrpS* and *hrpXY* is induced by conditions that mimic the apoplastic environment (Wei et al. 1992; this work). *hrpXY* shows high basal-level expression, and autoregulation is involved in gene induction. However, *hrpS* is not autoregulated based on results of the GUS assay, suggesting that there may be upstream regulatory components. Although *hrpS* provided in multiple-copy plasmids reverses the Hrp⁻ phenotype of *hrpX* and *hrpY* mutants, the independence of *hrpS* from *hrpX* and *hrpY* suggests that *hrpXY* is not epistatic to *hrpS* and environmental signals may go to *hrpS* through a different pathway.

Earlier work on *hrpL* and *hrpS* (Wei and Beer 1995) established that HrpS partially controls *hrpL* expression. Our current work indicates that the HrpX/HrpY system contributes to *hrpL* induction. Based on the role of *hrpXY* and *hrpS* in regulating *hrpL* and the lack of effect of *hrpX* and *hrpY* in *hrpS* expression, one might place *hrpS* upstream of *hrpXY*. This notion is precluded, however, because *hrpXY* does not override *hrpS* mutation. As mentioned above, the opposite is not likely, either. Therefore, it seems that signals independently perceived by *hrpXY* and *hrpS* converge at *hrpL*.

Neither HrpS nor HrpY alone induce high levels of *hrpL* expression, suggesting that cooperation of HrpY and HrpS, possibly through protein-protein interaction, may be needed for full activation of *hrpL*. In this model, HrpS may be a positive activator of *hrpL*, while HrpX/HrpY may act as a modulator of *hrpL* transcription. Complementation of *hrpX* and *hrpY* mutants for the HR phenotype by overexpressed *hrpS* supports this model. The regulation of *eps* genes of *R. solanacearum* seems similar; both VsrD and PhcA regulators bind to the *xpsR* promoter region and control *xpsR* expression (Huang et al. 1995b). In *P. syringae* pv. *syringae*, HrpR and HrpS have been proposed to work together to control *hrpL* expression (Xiao et al. 1994), although a different opinion exists for homologous proteins in *P. syringae* pv. *phaseolicola* (Grimm et al. 1995).

hrp gene regulation and Hrp phenotypes.

hrpY and *hrpS* seem to be crucial to the pathogenic life-style of *E. amylovora*, since their inactivation by mutagenesis results in loss of pathogenicity in immature pears (Wei et al. 1992; this work). The *hrpX* mutant, however, shows an attenuated phenotype: slightly lowered *hrpL* expression and reduced HR and virulence at higher inoculum doses. Currently, we cannot rule out the possibility of partial HrpX function in that mutant, even though leaky phenotypes of sensor mutants have been documented for other two-component systems (Stock et al. 1989). It is interesting to note that, although *hrpX* and *hrpS* mutants show different phenotypes (the former reduced Hrp and the latter Hrp⁻), both are similarly affected in *hrpL* expression, i.e., only three- to fourfold reduction. This suggests that either there is a threshold level of *hrp* gene expression required for causing disease, or *hrpS* is involved in expression of other genes that contribute to pathogenicity. Further study might distinguish between these two possibilities.

The incomplete complementation of *hrpX* and *hrpY* mutants by *hrpXY* provided in a multicopy plasmid at lower inoculum levels ($\leq 2 \times 10^3$ CFU per ml) is intriguing and deserves further investigation. One explanation for the results could be that defective HrpX and HrpY in the mutants interact with functional HrpX and HrpY, and, possibly by forming heterodimers, interfere with the full activity. Alternatively, overproduced HrpX and HrpY may somehow down-regulate *hrpS* expression.

Model of the *E. amylovora hrp* gene expression.

Based on previous studies (Bogdanove et al. 1996, 1998b; Kim and Beer 1998; Kim et al. 1997; Wei and Beer 1995; Wei et al. 1992) and results described in this work, we propose a scheme of *hrp* gene regulation in *E. amylovora* (Fig. 7). When the bacteria enter the plant apoplast, HrpX perceives environmental signals and is phosphorylated. Activated HrpX then phosphorylates HrpY to activate it, and increases the expression of *hrpXY* to produce more HrpX and HrpY. Independently, expression of *hrpS* is induced in response to the changed environment. Activated HrpY and HrpS, bound to the *hrpL* promoter, then interact with the RNA polymerase-σ^{54} complex to drive transcription of *hrpL*. HrpS also activates other genes containing the –24/–12 promoter consensus sequence. Finally, the HrpL σ factor, which recognizes a conserved promoter motif, GGAACC-N_{15}-CCACTAAT, directs transcription of the remaining *hrp* and *dsp* genes that produce the secretion machinery and virulence proteins that interact with plant cells.

MATERIALS AND METHODS

Bacterial strains and growth condition.

E. amylovora Ea321 is a wild-type strain that infects pear and apple (Beer et al. 1991). *Escherichia coli* DH5α was routinely used for cloning of cosmids and plasmids. pCPP1001 (Wei and Beer 1995), pCPP1036 (Kim et al. 1997), pCPP1078 (Wei and Beer 1995), and pCPP1178 are subclones of pCPP430 (Beer et al. 1991), and contain ORFs in the same direction as the T7Φ10 promoter from the vector pBluescript KS+. Strains of *E. amylovora* Ea321 and *Escherichia coli* were grown in LB (Sambrook et al. 1989) with vigorous shaking at 28 and 37°C, respectively. Inducing medium (IM) was used for inducing *hrp* gene expression as described previously (Wei et al. 1992). The antibiotics used to maintain selection were ampicillin at 100 μg/ml, kanamycin (Km) at 50 μg/ml, spectinomycin (Sp) at 50 μg/ml, tetracycline (Tc) at 20 μg/ml, and carbenicillin (Cb) at 300 μg/ml.

Recombinant DNA techniques and sequence analysis.

Unless otherwise specified, basic molecular biology techniques were as described (Sambrook et al. 1989). Electroporation of plasmid DNA into *E. amylovora* 321 and its derivatives was done as described by Bauer and Beer (1991) with the Gene Pulser apparatus (Bio-Rad, Richmond, CA, U.S.A.).

Deletion clones, generated from the *ClaI-BglII* insert in pCPP1178 with the Erase-A-Base kit (Promega, Madison, WI, U.S.A.), were sequenced by the dideoxy chain termination procedure with the Sequenase sequencing kit (U.S. Biochemical, Cleveland, OH, U.S.A.). Also, sequencing of the region between *hrpA* and *hrpJ* in pCPP430, pCPP1001, pCPP1036, and pCPP1178 was performed on an ABI 373A automated DNA sequencer (Perkin-Elmer, Norwalk, CT, U.S.A.) at the

Cornell University Biotechnology Program DNA Sequencing Facility with oligonucleotide primers synthesized at the same facility.

DNA and deduced amino acid sequences were analyzed with programs in the GCG software package, version 7.3 (Genetics Computer Group, Madison, WI, U.S.A.) and DNASTAR (DNASTAR, Madison, WI, U.S.A.). Potential genes were identified with GeneMark.hmm (Lukashin and Borodovsky 1998; available on-line from the GeneMark web site). Homology searches were done with BLAST algorithms (Altschul et al. 1997; available on-line from the NCBI web site). Conserved patterns in proteins were found with ScanProsite (Appel et al. 1994; available on-line). Finally, prediction of potential σ^{70} promoters were made with the Promoter Prediction by Neural Network method (Reese and Eeckman 1995; available on-line).

Expression of *hrpX* and *hrpY* in *Escherichia coli*.

A gene expression system mediated by a T7 RNA polymerase/promoter (Tabor and Richardson 1985) was used. pCPP1178, which contains *hrpX* and *hrpY* ORFs driven by the T7Φ10 promoter from the vector, was introduced into *Escherichia coli* DH5α(pGP1-2). Cells were incubated at 42°C to induce the expression of the T7 RNA polymerase gene, and newly synthesized proteins were radiolabeled with ^{35}S-Met as described (Tabor and Richardson 1985). Resulting samples were resuspended in a sample buffer and heated to 95°C for 3 min before being electrophoresed in a 12% polyacrylamide gel.

Construction of marker-exchange mutants.

Chromosomal mutants were constructed by marker-exchange mutagenesis as described previously (Wei et al. 1992). A Tn*10*-miniKm insertion or a Tn*phoA* insertion, mapped at the *hrpXY* or *hrpL* locus in *Escherichia coli* DH5(pCCPP430) or *Escherichia coli* DH5α(pCPP1178), was introduced into *E. amylovora* Ea321 by triparental mating with the helper strain, *Escherichia coli* HB101(pRK600) (kindly provided by E. R. Signer; Department of Biology, Massachusetts Institute of Technology, Cambridge). The transconjugants were selected on Luria plates containing Km and Sp, and then transferred to a low-phosphate minimal medium (Bauer 1990) to select for Km^r Sp^s marker-exchanged mutants. The second mutations were generated by introducing individual *hrp*::Tn*5-gusA1* fusions into Tn*10*-miniKm or Tn*phoA* mutants of Ea321. Since the transposon Tn*5-gusA1* has two selection marker, Km and Tc, the second mutation was selected based on Km^r Tc^r Sp^s phenotype. All the mutants were tested for the HR-eliciting ability and pathogenicity. Tn*phoA* insertions P74 and P86 in pCPP1036, which were mapped to *orfU1*, were introduced to the Ea321 genome by electroporation and subsequent incubation in a low-phosphate medium with Km. Integration of the Tn*phoA* fusion into the chromosome was confirmed by antibiotic resistance (Km^r Cb^r) and Southern hybridization with the transposon DNA as a probe.

Assay of GUS activity.

Overnight cultures in LB were transferred to fresh LB, and incubated further. For incubation in IM, log-phase cultures in LB were centrifuged, and cells were washed with IM, before they are resuspended in IM to $OD_{620} \approx 0.5$. The cultures in IM were incubated for an additional 5 to 6 h at 24°C before assay of GUS activity. GUS activity was monitored fluorimetrically as described by Jefferson et al. (1987). Forty-five microliters of the log-phase culture in LB or the induced culture from IM was mixed with an equal volume of 2× assay buffer. After incubation at 37°C for 10 h, GUS activity was measured as described previously (Wei et al. 1992). The background fluorescence of Ea321-G77 (*hrcV*::Tn*5-gusA1*) (Wei et al. 1992), which has a *gusA1* insertion in the opposite direction of *hrcV* transcription, was subtracted from the readings of *hrp*::*gusA1* fusion strains. The corrected fluorescence readings were converted to picounits of GUS activity per CFU. The GUS activity of *hrp*::Tn*5-gusA1* fusions also were determined in tobacco leaf tissues as described previously (Wei et al. 1992).

Plant assays.

Bacteria were grown in LB and harvested at mid-exponential phase. Cells were resuspended in 5 mM potassium phosphate buffer, pH 6.5, harvested again, resuspended in the potassium phosphate buffer to approximately 2×10^8 CFU per ml, unless otherwise specified, and used for HR and pathogenicity assays. Tobacco plants (*Nicotiana tabacum* L. 'Xanthi') were grown in greenhouse soil mix to a height of 0.9 to 1 m. Bacterial suspensions were infiltrated into each leaf panel of tobacco leaves with needleless hypodermic syringes. The development of the HR was scored after incubation at room temperature for 18 to 36 h. Pathogenicity tests on immature pear fruits were carried out as previously described (Bauer and Beer 1991; Steinberger and Beer 1988).

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NOTE ADDED IN PROOF

A recent BLAST survey of finished and unfinished microbial genomes (available on-line from the NCBI web site) suggests the presence in *Pseudomonas aeruginosa* PAO1 of a two-component system that is highly similar to the HrpX/HrpY system (31% identity over 474 amino acids for HrpX and 48% identity over 208 amino acids for HrpY). A related set of proteins exist in the *Pseudomonas putida* KT2440 genome.

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